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Research paper

Self-microemulsifying drug delivery system (SMEDDS) improves anticancer effect of oral 9-nitrocamptothecin on human cancer xenografts in nude mice

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Abstract

9-Nitrocamptothecin (9-NC) is an orally administered topoisomerase-I inhibitor for the treatment of pancreatic carcinoma, but its oral absorption and bioavailability are poor. The main objective of this study was to develop optimal 9-nitrocamptothecin (9-NC) microemulsion prepared by self-microemulsifying drug delivery system (SMEDDS). Two SMEDDS formulations of 9-NC prepared from a mixture of ethyl oleate, Tween-80 (T-form) or Cremophor EL (C-form), and PEG-400/ethanol were formed as microemulsions under dilution with aqueous phase. The resulting microemulsions were evaluated in vitro and in vivo, including the kinetics and antitumor effects in SKOV-3 human ovarian cancer xenograft in nude mice. Following 1:10 aqueous dilution of optimal 9-NC SMEDDS, the droplet sizes of resulting microemulsions were (30.8 ± 4.6) nm and (39.8 ± 8.2) nm for SMEDDS T-form and C-form, respectively, and the zeta potential values were $-(4.3 \pm 0.5)$ mV and $-(5.7 \pm 0.5)$ mV, respectively. In SKOV-3 cells, the growth inhibition (IC_{50}) of various 9-NC formulations was greatest with SMEDDS T-form (3.5 ± 0.7 nM) followed by SMEDDS C-form (4.6 ± 0.4 nM), 9-NC solution (6.6 ± 1.4 nM) and 9-NC suspension (26.0 ± 2.9 nM) ($P < 0.01$). It was indicated that the area under the plasma concentration–time curve ($AUC_{0\rightarrow 8\,h}$) values of various formulations of 9-NC after oral administration ranked as the following sequence: SMEDDS T-form (360.12 ± 19.44 ng h/ml) $\approx$ SMEDDS C-form (351.71 ± 33.66 ng h/ml) > 9-NC solution (241.21 ± 24.67 ng h/ml) > 9-NC suspension (161.24 ± 24.31 ng h/ml). The 9-NC SMEDDS formulations also produced significantly more tumor shrinkage ($P < 0.01$) when compared to 9-NC suspension in nude mice bearing human ovarian cancer xenografts. The results suggest that SMEDDS is a promising drug delivery system to increase the oral bioavailability and antitumor effects of 9-NC and may be applied to other lipophilic drugs. 9-NC SMEDDS represents a novel 9-NC therapy for cancer patients.

Keywords: 9-Nitrocamptothecin; Self-microemulsifying drug delivery system (SMEDDS); Pharmacokinetic; Antitumor activity

1. Introduction

For the past few years, 9-nitrocamptothecin (9-NC), a second-generation topoisomerase-I inhibitor and an analog of camptothecin (CPT), has been a focus of attention in cancer research with encouraging early clinical results [1–3]. 9-NC is an orally active agent being developed for the

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treatment of pancreatic cancer and other solid tumors by SuperGen [4]. Pharmacological studies have shown that the antitumor activity of 9-NC is superior to the activity of CPT in human tumors xenografted in nude mice [5]. Preclinically, 9-NC has also shown activity against a broad spectrum of tumor types both in vitro and in vivo human tumor xenograft models [4].

9-NC is practically insoluble in water and poorly absorbed from the gastro-intestinal (GI) tract [6]. To enhance the solubility and improve the bioavailability of 9-NC, researchers have tried oils [7], self-emulsifying formulations [8], or liposomes [9]. Recently, 9-NC liposome aerosol was developed and applied in the preclinical and clinical studies [10–12]. Knight et al. [11] have demonstrated the effectiveness of this liposomal formulation in the treatment of the human cancer xenografts in nude mice at doses much smaller than those traditionally used in mice administered by other routes.

Among these approaches, self-microemulsifying drug delivery system (SMEDDS) was chosen to evaluate lipophilic drugs such as 9-NC with poor water solubility and low bioavailability after oral delivery. SMEDDS is an isotropic mixture of oil, surfactant, cosurfactant (or coemulsifier), and drug. The basic principle of this system is its ability to form fine oil-in-water (o/w) microemulsions with gentle agitation following dilution by aqueous phases. In human, the digestive motility of the stomach and intestine provides the agitation required for self-emulsification in vivo [13,14]. The spontaneous formation of an emulsion upon drug release in the GI tract advantageously presents the drug as a dissolved form. In addition, the small droplet emulsion gives a size surface area for easy absorption [8,15]. To select a suitable self-emulsifying vehicle, it is mandatory to assess droplet size following self-emulsification [15]. In this study, two types of self-emulsifying formulations which named as SMEDDS C-form (Cremophor EL) and T-form (Tween-80) were prepared using oil (ethyl oleate), surfactants (Cremophor EL or Tween-80), and cosurfactant (PEG-400/ethanol = 3/1 (w/w)) for testing. Cremophor EL is a synthetic nonionic surfactant derived from polyethoxylated castor oil, while Tween-80 is a nonionic surfactant and emulsifier derived from polyoxylated sorbitol and oleic acid. Although both compounds can stabilize emulsions of nonpolar materials in aqueous systems, their ability to do so and the phase behaviors are different.

Our objectives were to develop and characterize the optimal formulations of SMEDDS containing 9-NC and to assess its bioavailability and antitumor activity compared with 9-NC suspension prepared with phosphate buffered saline (PBS) at pH 7.4 in animals.

2. Materials and methods

2.1. Materials

9-Nitrocamptothecin (9-NC, purity = 99.8%) was obtained from XieLi Pharmaceutical Technology Develop Co., (China). Cremophor EL was obtained from BASF (Ludwigshafen, Germany). Tween-80 and PEG-400 were purchased from Shandong Bangde Biotech (Qingdao, China) and BoDi Chemical Co. (Tianjin, China), respectively. Deionized water was prepared by a Milli-Q purification system from Millipore (Molsheim, France). Acetonitrile and methanol (HPLC grade, Burdick & Jackson, Morristown, NJ, USA) were used in the present study. All other chemicals were of reagent grade.

2.2. Preparation of 9-NC SMEDDS

The pseudo-ternary phase diagrams of oil, surfactant/cosurfactant, and water were developed to optimize the formulations with water titration method [13]: the mixtures of oil and surfactant/cosurfactant at certain weight ratios were diluted with water in a dropwise manner. SMEDDS T-form was developed from a mixture of oil (ethyloleat), surfactant (Tween-80), cosurfactant (PEG-400), and 9-NC. Cremophol EL instead of Tween-80 was used as surfactant for SMEDDS C-form. In these two SMEDDS formulations, the level of 9-NC was equal (i.e. 0.5% (w/w) of the vehicle). Briefly, 9-NC was firstly dissolved in the mixture of PEG-400 and ethanol (3:1, as cosurfactant) in glass vials, then oil and surfactant (Cremophor EL or Tween-80) were added into glass vials. After gentle stirring, SMEDDS formulation was formed and kept clear (detailed components seen in Table 1). The mixture was stored at room temperature until used. Before using it for in-vitro test, microemulsions were prepared by diluting SMEDDS with saline. The blank SMEDDS formulations were prepared by the same method as above without 9-NC.

2.3. Characterization of 9-NC microemulsions

Microemulsions were formed following 1:10 dilution of 9-NC SMEDDS (T-form and C-form) with distilled water, saline, and 5% of glucose solution. The droplet size and zeta potential of the microemulsions were determined by a Malven zetasizer Nano-ZS (Malven Instruments, Malven, UK). The stability of 9-NC microemulsions following 1:10 dilution with gastric and intestinal fluids was evaluated to rule out the possibility of 9-NC precipitation using optical imaging light microscope (Chongqing, China) equipped with an Olympus digital camera (Tokyo, Japan).

Table 1
Composition of SMEDDS formulations

Vehicle	T-form	C-form
9-NC (mg)	5.0	5.0
Ethyl oleate (mg)	250	250
PEG-400 (mg)	300	300
Tween-80 (mg)	345	/
Cremophor EL (mg)	/	345
Ethanol (mg)	100	100
Mean particle size (nm)	30.8 ± 4.6	39.8 ± 4.2
Zeta potential (mV)	$-(4.3 \pm 0.5)$	$-(5.7 \pm 0.5)$

2.4. Cell culture

The human ovarian carcinoma cell line SKOV-3 (HTB-77) was purchased from American Tissue Culture Collection (ATCC, Manassas, VA, USA), and cells were grown in RPMI 1640 medium supplemented with 10% of fetal bovine serum, 1% of L-glutamine, and 1% of penicillin/streptomycin (pH 7.4) in a humidified atmosphere containing 5% CO_2 at 37 °C. All cell culture reagents were purchased from Gibco BRL (Beijing, China). Human neuroblastoma cell line (SH5Y), human breast cancer cell line (MCF-7), and human breast cancer cell line (MDA-MB-231) were kindly provided by Prof. Cui in Peking University (Beijing, China).

2.5. In-vitro cytotoxicity of 9-NC SMEDDS

Since 9-NC was practically insoluble in water, its solution was prepared by dissolving 9-NC in polyethylene glycol (PEG-400)/dimethyl sulfoxide (DMSO) (20/1, v/v) mixture solvent and then diluted with water. 9-NC suspension was prepared by milling 9-NC powder with 1.0% of carboxymethylcellulose sodium (CMC-Na) solution. In-vitro cytotoxicities of 9-NC SMEDDS (T-form and C-form) on human cancer cell lines were determined by the sulforhodamine B (SRB) assay [16]. A total of four cancer cell lines, including human neuroblastoma cell line (SH5Y), human epithelial ovarian cancer (SKOV-3), human breast cancer (MCF-7), and human breast cancer (MDA-MB-231) were included in the cytotoxicity study. In brief, 5×10^3 cells were seeded in each well in a 96-well culture plate. Twenty-four hours later, fresh medium containing a series of concentrations of various test compounds including 9-NC SMEDDS T-form, 9-NC SMEDDS C-form, 9-NC solution and 9-NC suspension were added. Cells incubated in the fresh medium containing blank SMEDDS vehicles (T-form and C-form) were included as controls. After 72 h of incubation, the IC_{50} value was calculated as the concentration of 9-NC that gives a 50% cell growth inhibition. 9-NC SMEDDS formulations were prepared according to the method mentioned above and diluted with 1:10 fresh medium before addition to culture plates. 9-NC solution dissolved in DMSO and 9-NC suspension prepared with PBS at pH 7.4 were also tested. The concentration of 9-NC in various preparations ranged from 0.5 to 100 nM.

2.6. HPLC assay of 9-NC

The concentration of 9-NC in the samples was determined by HPLC analysis modified from a previous study [17]. The SHIMADZU LC-10A HPLC analysis system equipped with an ultraviolet detector and a Phenomenex Luna C18 column of 4.6 mm × 250 mm (5 μm, Torrance, CA, USA) was used. A mobile phase of acetonitrile:water (38:62, adjusted to pH 3.5 by glacial acetic acid) was pumped isocratically at a flow rate of 1.0 ml/min. A 40 μl volume was injected into the column and the effluent was monitored at 368 nm. The column temperature was set to 40 °C.

For analysis, the frozen plasma was thawed prior to liquid–liquid extraction procedure at room temperature. Briefly, an aliquot of 100 μl internal standard (SN-38, 150 ng/ml in methanol), 100 μl 0.1 M hydrochloric acid and 100 μl methanol were added to 200 μl rat plasma in a 10 ml polypropylene tube. The mixture was vortex-mixed for 1 min, then 3.0 ml of extract solution (*N*-hexane–dichloromethane–isopropyl alcohol, v/v/v = 100/50/5) was added. The mixture was mixed for 3 min and was subsequently centrifuged for 10 min at 10,000 rpm at room temperature. The supernatant was collected in a glass tube and evaporated at 40 °C under a gentle stream of nitrogen gas, until a completely dried residue was left over. The residue was reconstituted in 100 μl of the mobile phase by vortexing and a 50 μl volume was injected into the HPLC.

In the concentration range of 10–2000 ng/ml, good correlation coefficient was observed between peak area ratio of 9-NC/internal standard and 9-NC concentration ($r^2 = 0.9991$, $n = 6$). The limit of quantification and the limit of detection ($S/N > 3$) were 10.0 and 5.0 ng/ml, respectively. At concentrations of 10.0, 100.0, and 1000.0 ng/ml, the recoveries of 9-NC from rat plasma were 101.65%, 103.51%, and 99.35%, respectively. The intra-day precision was 10.38%, 8.79%, and 3.21%; and inter-day precision was 11.58%, 6.82%, and 2.87%, respectively. After storage for 1 month at −20 °C and freeze-thawing for three times, 9-NC was stable in plasma.

2.7. Bioavailability studies

Healthy male Sprague–Dawley rats weighing 200–250 g were used in the pharmacokinetic study. The animals were fasted overnight with free access to water. The experimental procedure was approved by the Committee on Use and Care of Animals at the Peking University (Beijing, China). Animals were allocated at random to three treatment groups. The rats ($n = 6$ per group) were administered with a single dose of 9-NC solution (6 mg/kg), SMEDDS T-form (6 mg/kg), and SMEDDS C-form (6 mg/kg) according to the experimental protocol. All formulations were administered orally with a 21-gauge catheter. Another six rats were administered intravenously with a 9-NC injection. Blood samples (0.5 ml) were collected from orbital venous into the heparinized tubes at the following time points immediately before administration, 5 min, 15 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 3 h, 5 h, and 8 h. Blood samples were centrifuged at 3000 rpm for 30 min and the resultant plasma was transferred into capped tubes and stored at −20 °C until analysis.

2.8. In-vivo antitumor activity

Nude mice bred and housed at the Animal Experimental Centre of Peking University were used for the antitumor experiments. Approximately 5×10^6 tumor cells in 0.5 ml

Eagle's modified essential medium was injected under the skin over the right dorsal chest region. The animals were divided into three groups ($n = 6$ per group) and started on treatment with the experimental drug (9-NC suspension, SMEDDS C-form and SMEDDS T-form) at the dose of 6 mg 9-NC/kg about 5 days after implantation of tumors. The sizes of tumors in the study were measured in two dimensions (area) with calipers every day.

2.9. Data analysis

The pharmacokinetic parameters were performed by a non-compartmental analysis using WinNonlin 3.3® pharmacokinetic software (Pharsight, Mountain View, CA, USA). The area under the concentration–time curve ($AUC_{0\rightarrow 8\,h}$) was estimated according to the trapezoidal rule. The relative bioavailability (BA) of SMEDDS formulation (T) to the 9-NC suspension (R) was calculated using the following equation:

$$\text{Relative BA}(\%) = \frac{AUC_T}{AUC_R} \times \frac{Dose_R}{Dose_T}$$

.

Data are presented as means ± SD and where applicable, the differences among groups were analyzed by the Student's *t*-test and one-way ANOVA. A *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. Preparation and characterization of 9-NC SMEDDS

Pseudo-ternary phase diagrams were constructed to identify the self-emulsifying regions and to optimize the SMEDDS formulations of 9-NC. In the diagrams consisting of oil, surfactant (S)/cosurfactant (CoS) and water, different S/CoS ratios (K_m), 1/9, 2/8, 3/7, 4/6, 5/5, 6/4, 7/3, 8/2, and 9/1, were screened under the condition of fixed oil proportion (50%). With the decreasing of S/CoS ratios (K_m), the area of self-microemulsifying region decreased slightly. Self-microemulsifying formulations could be formed within the K_m values of 4/6, 5/5, and 6/4. The optimal formulation of 9-NC SMEDDS was selected according to self-microemulsifying ability, solubilization ability and reduced proportion of surfactant ($K_m = 0.86$), as shown in Table 1. The two types of microemulsions were obtained following 1:10 aqueous dilution of SMEDDS which contained constant concentrations of 0.6% 9-NC, 25% oil (ethyl oleate), 34.5% surfactant (Tween-80 or cremophor EL) and 40% cosurfactant (PEG-400/ethanol = 3/1 (w/w)). Also, it has been shown that the resultant microemulsions remained physically stable for at least 12 h without seeing any 9-NC precipitation.

As shown in Table 1, the droplet sizes of the microemulsions were (30.8 ± 4.6) nm and (39.8 ± 8.2) nm, respectively, following a 1:10 aqueous dilution of the optimal SMEDDS T-form and C-form. The resulting microemulsions were negatively charged, and the zeta potential values were −(4.3 ± 0.5) mV and −(5.7 ± 0.5) mV for T and C forms, respectively. The pH values of the microemulsions were approximately 7.0. Morphology and particle size distribution of microemulsions following a 1:10 dilution of SMEDDS T-form and C-form containing 6 mg/ml 9-NC with saline were shown in Fig 1. There was no aggregation or adhesion among droplets of microemulsion.

Following a 1/10 dilution with artificial gastric acid (pH 2.0) and artificial intestinal fluids (USP XXIII), 9-NC microemulsion remained physically stable for at least 12 h without observing 9-NC precipitation. No precipitation from microemulsions was observed following aqueous dilution of 9-NC SMEDDS for over 6 months.

3.2. In-vitro growth inhibition

The growth inhibition of four kinds of human cancer cells treated with 9-NC solution, 9-NC suspension, SMEDDS T-form, and SMEDDS C-form is shown in Table 2. The IC_{50} of 9-NC solution for four cells was in an order of MDA-MB-231 (IC_{50}: 33.3 ± 1.7 nM) > SH5Y (IC_{50}: 11.0 ± 1.5 nM) > SKOV-3 (IC_{50}: 6.6 ± 1.4 nM) > MCF-7 (IC_{50}: 3.7 ± 1.3 nM), which indicated different sensitivity of human cancer cells to 9-NC ($P < 0.05$). In SKOV-3 cells, the growth inhibition (IC_{50}) of various 9-NC formulations was greatest with SMEDDS T-form (IC_{50}: 3.5 ± 0.7 nM) followed by SMEDDS C-form (IC_{50}: 4.6 ± 0.4 nM), 9-NC solution (IC_{50}: 6.6 ± 1.4 nM) and 9-NC suspension (IC_{50}: 26.0 ± 2.9 nM) ($P < 0.01$). Interestingly, the growth inhibition of four human cancer cells treated with SMEDDS T-form was not significant by different from that of C-form ($P > 0.05$), and both were significant by higher than that of 9-NC suspension and solution ($P < 0.01$) (Table 2). In our results, the cell growth inhibitions of the two blank vehicles (SMEDDS T-form and C-form) were less than 10% within the range of dilution.

3.3. Pharmacokinetic study of 9-NC SMEDDS

Based on the self-emulsification properties, particle size data, and stability of microemulsion, two formulations of 9-NC SMEDDS (T-form and C-form) were selected for bioavailability studies. The in-vivo study was performed to quantify 9-NC after administration of 9-NC formulations. The plasma profiles of 9-NC in healthy Sprague–Dawley rats following oral administration of the 9-NC suspension and SMEDDS formulations were compared. Fig 2 shows that the plasma concentration profiles of 9-NC from SMEDDS were significantly different from those of the 9-NC suspension. Pharmacokinetic parameters of the maximum plasma concentration (C_{max}) and the corresponding time (T_{max}) for 9-NC following oral administration are shown in Table 3.

From Table 3, it was shown that the $AUC_{0\rightarrow 8\,h}$ values of various formulations of 9-NC ranked as the following sequence: SMEDDS T-form (360.12 ± 19.44 ng h/ml) ≈

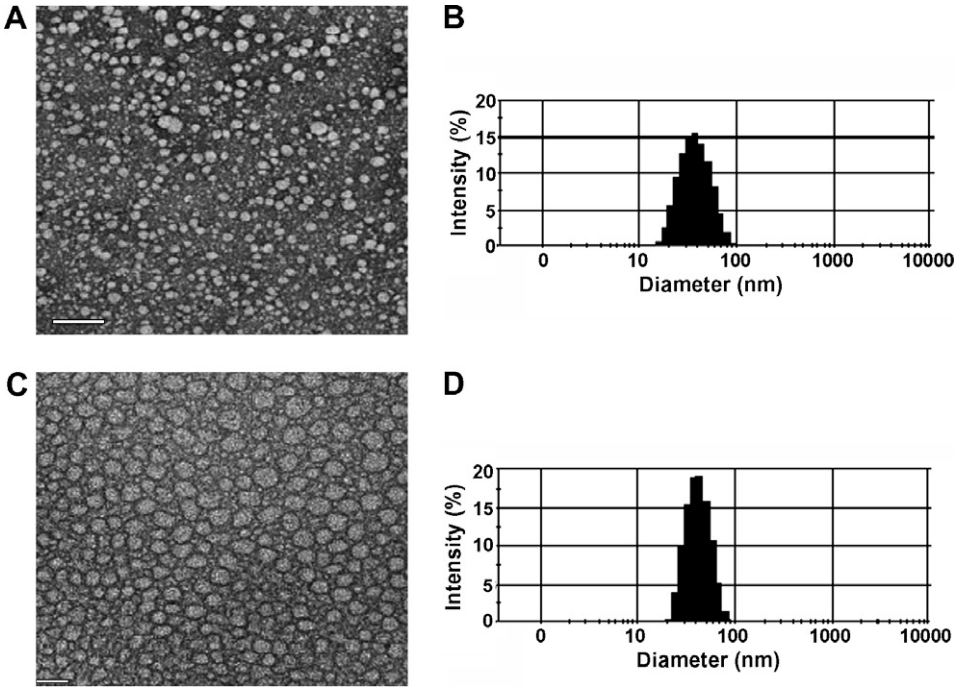


Fig. 1. Typical morphologies (A and C) and particle size distribution diagrams (B and D) of microemulsion following 1:10 dilution of SMEDDS T-form and C-form containing 6 mg/ml 9-NC with saline (The bars of A and C represent 200 nm and 50 nm, respectively; and the average particle sizes of B and D are 30.8 and 39.8 nm, respectively).

Table 2
Cytotoxicity (IC_{50}) of various 9-NC formulations against four human tumor cell lines ($n = 6$)

Formulation	IC_{50} (nM)			
	SH5Y	SKOV-3	MCF-7	MDA-MB-231
SMEDDS (T-form)	7.0 ± 0.6	3.5 ± 0.7	1.3 ± 0.6	21.8 ± 3.2
SMEDDS (C-form)	6.8 ± 0.8	4.6 ± 0.4	2.1 ± 1.0	24.1 ± 0.7
9-NC suspension	53.5 ± 5.9	26.0 ± 2.9	6.9 ± 0.9	43.5 ± 0.7
9-NC solution	11.0 ± 1.5	6.6 ± 1.4	3.7 ± 1.3	33.3 ± 1.7

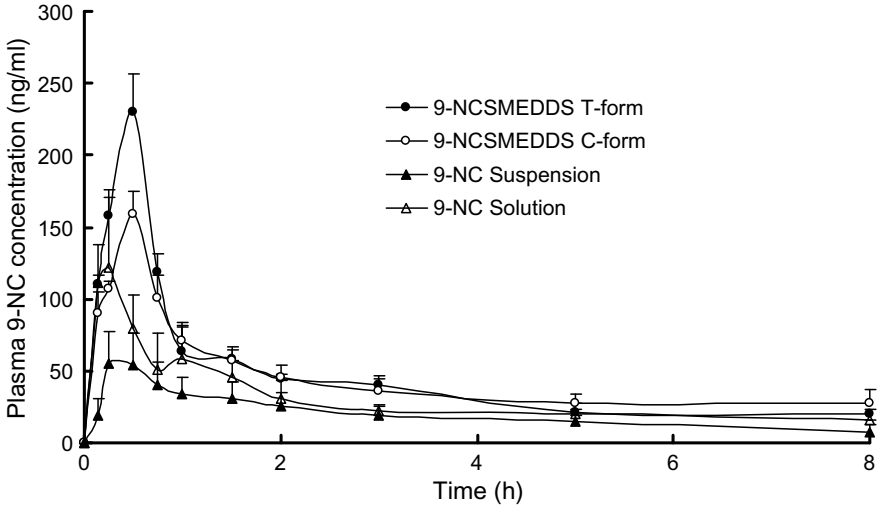


Fig. 2. Plasma concentration–time profiles of 9-NC after oral administration of the 9-NC suspension (-▲-), 9-NC solution (-△-), SMEDDS T-form (-●-), and C-form (-○-) at the single dose of 6 mg/kg in healthy Sprague–Dawley rats ($n = 6$).

SMEDDS C-form (351.71 ± 33.66 ng h/ml) > 9-NC solution (241.21 ± 24.67 ng h/ml) > 9-NC suspension (161.24 ± 24.31 ng h/ml). The C_{max} values of T-form, C-form, solution, and suspension of 9-NC were 229.60 ± 12.50 ng/ml,

Table 3
Pharmacokinetic parameters of 9-NC after various formulations were orally administered (6 mg/kg) into healthy rats ($n = 6$, mean ± SD)

Parameters	Oral				IV
9-NC formulations	Suspension	SMEDDS C-form	SMEDDS T-form	Solution	9-NC Solution[d]
C_{max} (ng/ml)	62.97 ± 8.64	159.45 ± 15.81[a,c]	229.60 ± 12.50[a,b,c]	132.15 ± 41.97	346.86 ± 112.92
T_{max} (h)	0.33	0.5	0.5[a,c]	0.17	/
CL (ml/h)	6552.02 ± 1993.84	2168.1 ± 492.67[a]	2827.0 ± 228.26[a,b]	3007.81 ± 980.92	74.02 ± 0.18
MRT (h)	2.81 ± 0.46	2.93 ± 0.26	2.44 ± 0.11[b]	2.71 ± 0.26	0.99 ± 0.17
$t_{1/2}$ (h)	3.3 ± 0.9	6.3 ± 3.1[a]	3.9 ± 0.2[b]	3.5 ± 0.7	2.2 ± 0.6
$AUC_{0-8\,h}$ (ng h/ml)	161.24 ± 24.31	351.71 ± 33.66[a,c]	360.12 ± 19.44[a,c]	241.21 ± 24.67	237.45 ± 50.97
Absolute BA (*F%*)	16.98 ± 2.56	37.03 ± 3.54[a,c]	37.91 ± 2.05[a,c]	26.72 ± 2.17	/

BA, bioavailability; 9-NC, 9-nitrocamptothecin.
[a] $P < 0.01$ vs 9-NC suspension.
[b] $P < 0.01$ vs SMEDDS C-form.
[c] $P < 0.01$ vs 9-NC solution.
[d] 9-NC was dissolved in the vehicles as TAXOL® formulation and diluted with 5% glucose.

159.45 ± 15.81 ng/ml, 62.97 ± 8.64 ng/ml, and 132.15 ± 41.97 ng/ml, respectively. The comparable values of T_{max} of SMEDDS C-form and T-form indicated a similar onset of drug absorption. However, SMEDDS C-form and T-form microemulsions had a delayed absorption (T_{max} = 0.5 h) after oral administration when compared to 9-NC suspension (T_{max} = 0.33 h) and solution (T_{max} = 0.17 h). SMEDDS C-form and T-form microemulsions gave 2.18- and 2.23-fold increases of 9-NC oral bioavailability compared with the 9-NC suspension, respectively ($P < 0.01$); and SMEDDS microemulsions significantly enhanced the values of $AUC_{0\to 8\,h}$ and C_{max} of 9-NC compared with the 9-NC suspension and solution.

3.4. Antitumor activity

Various formulations (blank SMEDDS T-form, blank SMEDDS C-form, 9-NC suspension, 9-NC SMEDDS T-form, and 9-NC SMEDDS C-form) were orally administered at a 4-day interval from the 5th day post-inoculation. The photographs and growth rate (%) of tumors are shown in Fig 3. All of the 9-NC formulations were effective in inhibiting tumor growth compared to saline and blank vehicles ($P < 0.01$). Treatments with SMEDDS formulations showed stronger tumor growth inhibition than the treatment with 9-NC suspension ($P < 0.01$). However, no significant difference was observed between treatments with 9-NC SMEDDS T-form and C-form. Also, the two blank vehicles had no inhibition on tumor growth, as saline group.

3.5. In-vivo toxicity

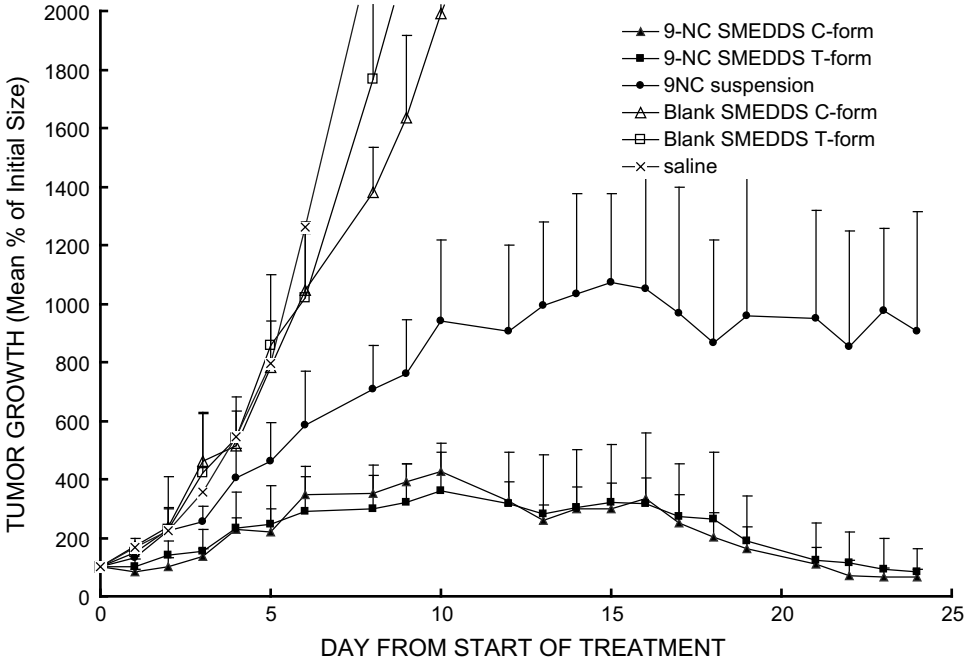
For safety purpose, we examined the important organs of a number of mice involved in this study, including heart, liver, spleen, lung, kidney, brain, and muscle, 24 days after the last treatment. None of the histological sections showed evidence of drug toxicity. Meanwhile, the changes of body weight from all the treated mice were examined and slight systemic toxicity was found in the groups treated with the formulations containing 9-NC (Fig 4), suggesting good toxicity profiles of 9-NC SMEDDS.

4. Discussion

Up to now, 9-NC has been administered to patients mainly by the oral route because it was significantly better than i.v. route in pharmacological study [5]. The pharmacokinetic parameters of oral administration of 6 mg/kg 9-NC solution showed the absolute bioavailability of lactone and total 9-NC were 23.4% and 22.7%, respectively, of that by intravenous injection. This indicated that lactone and total 9-NC were poorly absorbed following oral administration. In our study (Table 3), the pharmacokinetic profiles of 9-NC suspension have also shown a low absolute bioavailability of total 9-NC (about 17%) via oral administration.

Self-microemulsifying drug delivery system (SMEDDS) was used to improve the oral bioavailability of lipophilic drugs with poor water solubility and low bioavailability [13,18,19]. SMEDDS forms fine oil–water emulsions with only gentle agitation, upon its introduction into aqueous media. Since the free energy required to form an emulsion is very low, the formation is thermodynamically spontaneous. Surfactants form a layer around the emulsion droplets and reduce the interfacial energy as well as provide a mechanical barrier to coalescence. In the current study, the pseudo-ternary phase diagram was constructed in the presence of 9-NC to obtain the optimal SMEDDS containing ethyl oleate (oil), cremophor EL (or Tween-80) (surfactant) and PEG-400/ethanol (cosurfactant). We found that incorporation of 9-NC in SMEDDS significantly improves the solubility of 9-NC in artificial gastric acid and artificial intestinal fluids. After dilution, nanoscale (<100 nm) droplets were found in aqueous media using the transmission electron microscopy and there was no aggregation or adhesion. Also, there was no precipitation of 9-NC observed when the SMEDDS were diluted in the cell culture media and the phosphate buffer solution for over 12 h.

As in the previous study [5], 6 mg/kg of 9-NC was given orally for the pharmacokinetic and pharmacodynamic



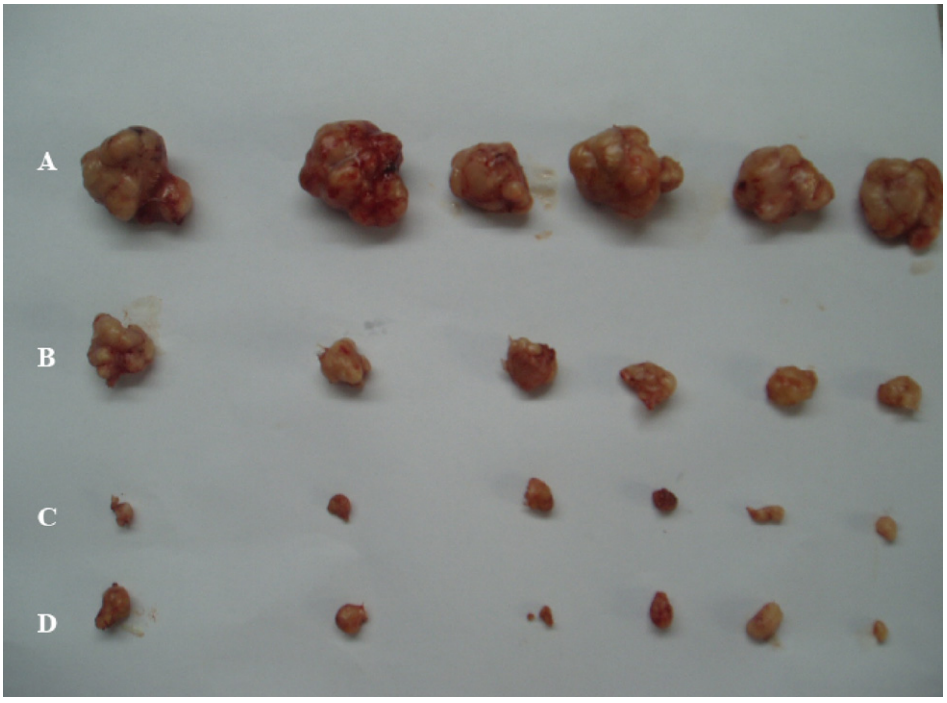


Fig. 3. Antitumor activities (growth inhibition ratio) of 9-NC suspension, and 9-NC SMEDDS T-form and C-form in nude mice bearing human ovarian SKOV-3 tumor after multiple oral administration at a dose of 6 mg/kg 9-NC (given every 4 days, as indicated by the arrows). Saline was used as the control, and the effects of blank vehicles of SMEDDS T-form and C-form were also investigated. Tumor size was measured for each animal everyday starting from the day of the initial treatment. Results are given as means ± SD ($n = 6$). Photographs of the SKOV-3 tumor treated with three doses of saline (A), 9-NC suspension (B), 9-NC SMEDDS T-form (C), and 9-NC SMEDDS C-form (D) orally for 30 days post-inoculation.

studies. In our results (Table 3), the absolute bioavailability of oral 9-NC suspension was only 16.98%. Compared to 9-NC suspension, higher oral bioavailability (26.72%) was found in 9-NC solution ($P < 0.01$). It suggested that improved dissolution may contribute to the enhancement of bioavailability of 9-NC. For SMEDDS formulations (T-form and C-form), significant enhancement of oral bioavailability (37.03% and 37.91%) was observed in rats compared with 9-NC suspension and solution. SMEDDS was shown to be a promising approach for increasing absorption by oral route of 9-NC and could increase bioavailability for the other poorly water-soluble drugs. We found that

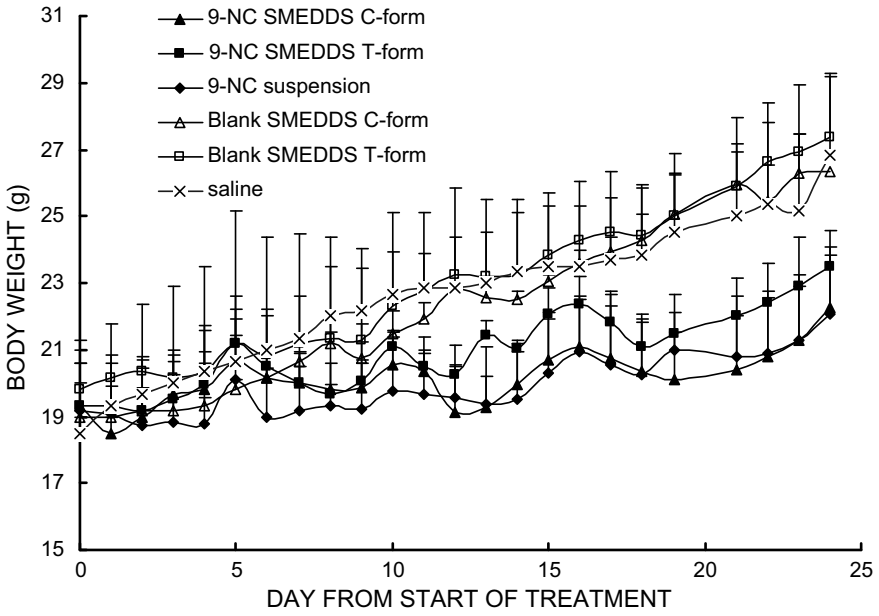


Fig. 4. The changes of body weight after orally administered with various 9-NC formulations (suspension, and SMEDDS T-form and C-form) at a dose 6 mg/kg on 0 day, 4th day and 8th day, or blank vehicles (SMEDDS T-form and C-form) and saline in nude mice. Body weight was measured for each animal everyday starting from the day of the initial treatment. Results are given as means ± SD ($n = 6$).

incorporation of 9-NC in SMEDDS significantly improved the oral bioavailability of 9-NC and resulted in a 2.18-fold (C-form) and 2.23-fold (T-form) increases, respectively, compared to the 9-NC suspension after oral administration ($P < 0.01$). It was rational to deduce that alternative mechanisms other than improving dissolution or release may result in enhancement of oral bioavailability of 9-NC. Interestingly, a delayed T_{max} was seen in the two SMEDDS formulations. Therefore, it is possible that a higher bioavailability of SMEDDS may result form enhanced absorption through the lymphatic pathway as reported previously [13,20]. The authors proposed a mechanism of M cell-mediated transport in intestinal tract that was rich in Peyer's patch and thus improved drug absorption through lymphatic pathway. Besides, high concentration of surfactants in SMEDDS may also result in an increase of permeability by disturbing the cell membrane [21]. It was supported from the comparison of cytotoxicity results of various formulations used in four kinds of human tumor cells (Table 2). Furthermore, the C_{max} values of SMEDDS T-form and C-form were 229.60 ± 12.50 and 159.45 ± 15.81 ng/ml after oral administration (Table 3), respectively. This difference might be explained by emulsification rate, mean particle size and absorption enhancement of microemulsion from SMEDDS T-form (mean droplet size: 30.8 nm) and C-form (mean droplet size: 39.8 nm) which contained different surfactants. The spontaneous formulation of an emulsion upon drug release in the GI tract effectively presents the drug in a solubilization form, and the small droplet size would provide a large interfacial surface area for drug absorption [8]. Interestingly, the values of $AUC_{0\rightarrow 8\,h}$ of SMEDDS T-form and C-form were similar (360.12 ± 19.44 ng h/ml and 351.71 ± 33.66 ng h/ml), but the $t_{1/2}$ values were 3.9 and 6.3 h, respectively. This might be explained by the fact that cremophor EL could affect the drug distribution and alter blood protein and tissue binding of the drug [22].

This study showed a potent anticancer effect of 9-NC SMEDDS formulations when they were orally administered to nude mice implanted with human ovarian cancer xenografts (SKOV-3). 9-NC SMEDDS had a statistically significant superiority in reducing tumor size to the control groups treated with 9-NC suspension ($P < 0.01$). In all mice examined, no significant toxicity was observed, indicating that 9-NC SMEDDS was well tolerated.

5. Conclusion

We have developed novel SMEDDS for the oral delivery of 9-NC. We demonstrated the SMEDDS formulations containing ethyl oleate as an oil phase, cremophor EL or Tween-80 as surfactant and PEG-400/ethanol as cosurfactant increased the solubility, bioavailability and antitumor activity of 9-NC significantly. SMEDDS is a promising delivery system for the safe and effective delivery of poor oral bioavailability drugs, such as 9-NC, with potential for application to human therapy by increasing the efficiency of oral administration.

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